

***Westerdykella aquatica* sp. nov., producing phytase**

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ABSTRACT—*Westerdykella aquatica*, isolated from rice field mud and as an endophyte from *Acorus calamus*, is described as a new species. The fungus is characterized by its globose cleistothecia, globose to subglobose, persistent, 8-spored asci, and cylindrical, 3-septate, light brown ascospores. A phylogenetic tree based on ITS, LSU, and β -tubulin sequences was constructed to infer the phylogenetic relationship between *W. aquatica* and other *Westerdykella* species in the genus. The new species was found to produce phytase, an important enzyme to release organic phosphorus in the soil.

KEY WORDS—Ascomycota, Dothideomycetes, freshwater fungi, phylogeny, *Sporangiaceae*

Introduction

Westerdykella Stolk was established to accommodate *W. ornata*, isolated from mangrove mud on the island of Inhaca, Mozambique, East Africa.

The genus is characterized by its globose, black and astomatous ascomata, membranous cleistothecium wall consisting of one layer of brown to black thick-walled cells, subglobose to elliptical multi-spored asci, and globose to subglobose, brown ascospores (Stolk 1955).

Currently, 13 additional species are accepted in *Westerdykella*: *W. angulata* (A.C. Das) Kruys, *W. aurantiaca* (J.N. Rai & J.P. Tewari) Kruys, *W. capitulum* (Panwar & al.) Gruyter & al., *W. centenaria* Crous & al., *W. cylindrica* (Malloch & Cain) Arx, *W. dispersa* (Clum) Cejp & Milko, *W. globosa* (J.N. Rai & J.P. Tewari) Tad. Ito & Nakagiri, *W. minutispora* (P.N. Mathur) Gruyter & al., *W. multisporea* (Cain) Cejp & Milko, *W. nigra* (Routien) Arx, *W. ornata* Stolk, *W. purpurea* (Cain) Arx, and *W. reniformis* Ebead & Overy (von Arx 1973, 1975, Cejp & Milko 1964, Crous & al. 2017, Ebead & al. 2012, Gruyter & al. 2013, Ito & Nakagiri 1995, Kruys & Wedin 2009, Stolk 1955).

Molecular phylogenies inferred from multi-loci showed that *Westerdykella* is monophyletic (Kruys & Wedin 2009; Ebead & al. 2012; Crous & al. 2017). However, morphologically some species do not fit well within the original generic concept (Stolk 1955). Therefore, a revision is needed to make the generic concept consistent with the species characters, and the asexual concept of the genus should be added.

Westerdykella species are an important bioresource, producing various bioactive substances such as antibiotics (Ebead & al. 2012; Xu & al. 2017b), cytotoxics (Xu & al. 2017a), enzyme inhibitors (Lee & al. 1997), and apoptosis inhibitors (Lee & al. 1999). During a long-term investigation of aquatic fungi (Hu & al. 2007, 2012a,b, 2017; Huang & al. 2018; Song & al. 2018a,b), a new *Westerdykella* species producing phytase was encountered in the mud of a rice field and also isolated as an endophyte of an *Acorus* plant in Jiangxi Province, China.

Phosphorus, an essential nutrient for the growth and development of living organisms, plays a vital role in virtually every plant process that involves energy transfer. Most organic phosphorus is in the form of phytic acid, an unavailable form of phosphorus. Phytase is an enzyme that releases the phosphorus from the phytate complex to make it available (Sandhya & al. 2015). The enzymatic degradation of phytic acid, which does not produce toxic by-products, is an environmentally friendly process (Ciofalo & al. 2003). In view of increasing demand, the production of phytase in a cost-effective manner using microorganisms is an important field of study (Shivanna & Venkateswaran 2014, Song & al. 2019). To the best of

our knowledge, this is the first paper assessing the phytase activities of the genus *Westerdykella*, a new bioresource of this enzyme.

Materials & methods

Sample collection and fungal isolation

Soil and plant samples were collected in various freshwater habitats in Jiangxi Province, China. The samples were placed in sterile ziplock plastic bags (Supin™) and brought back to the laboratory.

TABLE 1. Strains used in the phylogenetic analyses.

SPECIES	STRAIN No.	GENBANK ACCESS. NO.		
		ITS	28S	β-tubulin
<i>Forliomyces uniseptatus</i>	MFLUCC 15-0765	KU721772	KU721762	—
<i>Preussia funiculata</i>	Huhndorf 2577	GQ203762	GQ203722	GQ203685
<i>P. typharum</i>	CBS 107.69	GQ203766	GQ203726	GQ203689
<i>P. vulgaris</i>	Strid 18884	GQ203767	GQ203727	GQ203690
<i>Sparticola juncei</i>	MFLU 16-0242	KY659562	KY659565	—
<i>Sporormia fimetaria</i>	Lundqvist 2302-c	GQ203768	GQ203728	GQ203691
	UPS: Dissing Gr.81.194	GQ203769	GQ203729	GQ203692
<i>Sporormiella irregularis</i>	Lundqvist 16568-f	GQ203780	GQ203739	GQ203700
<i>S. leporina</i>	MJR93/04	GQ203782	GQ203741	GQ203702
<i>S. vexans</i>	UME23	GQ203793	GQ203751	GQ203712
<i>Westerdykella angulata</i>	CBS 610.74	GQ203757	DQ384105	—
	IMI 090323	GQ203758	GQ203720	GQ203680
<i>W. aquatica</i>	JAUCC 1788 [T]	MH411093	MH411090	MH424907
	JAUCC 0138	MH411092	MH411091	MH424906
<i>W. aurantiaca</i>	FNBR-03	JN118571	—	—
	IMI 086825	AY943057	—	—
<i>W. centenaria</i>	CBS 142400	KY979734	KY979790	KY979908
	CBS 272.74	KY979735	—	—
<i>W. cylindrica</i>	ATCC 24077	AY943056	AY004343	Jx235707
<i>W. dispersa</i>	CBS 156.67	DQ468016	—	—
	CBS 297.56	GQ203797	GQ203753	GQ203716
	CBS 508.75	GQ203798	DQ384099	—
<i>W. globosa</i>	IFO 32588	AY943046	—	—
	SWC5	KY065369	—	—
	SWC6	KY260681	—	—
<i>W. minutispora</i>	CBS 509.91	-	GU238108	—
<i>W. multisporea</i>	CBS 383.69	GQ203799	GQ203754	GQ203717
<i>W. nigra</i>	ATCC 12756	AY943049	—	—
	CBS 416.72	GQ203800	GQ203755	GQ203718
<i>W. ornata</i>	CBS 379.55	GQ203801	AY853401	GQ203719
<i>W. reniformis</i>	DAOM 242243	Jx235700	Jx235704	Jx235706
<i>Stemphylium vesicarium</i>	ATCC 11681	AF229479	AF382386	AY749032

Bold font indicates novel sequences generated in this study.

The soil samples were serially diluted up to 10^{-5} with sterile distilled water and spread on the surface of potato dextrose agar media (PDA) in plates, which were then incubated at 25 °C for 1–2 days. Stems of *Acorus calamus* were sliced into small lengths and cleaned 3–5 times with distilled water (Nonaka & al. 2013). Plant tissue pieces (c. 0.5×0.5 cm) were soaked in 75% alcohol for 30 seconds and immediately washed 3–5 times in sterile water, dried in a sterile fume hood for c. 5 minutes, and placed on PDA medium. Mycelium growing around the tissue was transferred to new PDA plates (Sati & al. 2009). Colonies with different morphological characters were transferred to new PDA plates. Cultures were dried as specimens and deposited in the Herbarium of Fungi, Jiangxi Agricultural University, Nanchang, China (HFJAU). Living cultures were deposited in the Culture Collection of Jiangxi Agricultural University, Nanchang, China (JAUCC).

Morphological studies

Strains were grown on PDA at 25 °C and their growth rates and colony morphology were evaluated after 7 and 14 days of incubation. The diameters of colonies were measured in five directions and the average values were applied to reflect the dimensions. For micro-morphological measurements and photographs, fungal structures from 30-day-old cultures were mounted in water on glass slides; and examined and photographed using a Nikon (Ni) compound microscope with differential interference contrast (DIC) and a dissecting microscope (Hu & al. 2017).

DNA extraction and PCR amplification

Genomic DNA of strains was extracted by CTAB (hexadecyl trimethyl ammonium bromide) method (Wu & al. 2001). Fragments of the partial large subunit (LSU), internal transcribed spacer (ITS) rDNA, and β -tubulin gene (Bt2) were amplified by the polymerase chain reaction (PCR). For PCR amplification, primer pairs LROR/LR6 were used for LSU (Rehner & Samuels 1995; Vilgalys & Hester 1990), ITS4/ITS5 for ITS (White & al. 1990), and T1/Bt2b for β -tubulin (Glass & Donaldson 1995, O'Donnell & Cigelnik 1997). The amplification was performed following the method described by Hu & al. (2012b). The PCR products were purified and sequenced with the same primers used for PCR in a sequencer (ABI-PRISM3730) at Tsingke Biological Technology Company, Beijing.

Phylogenetic analyses

Six novel sequences from the two strains of the new taxon (JAUCC 1788, JAUCC 0138) and reference sequences obtained from GenBank (TABLE 1), were aligned with MAFFT v.7 (<https://mafft.cbrc.jp/alignment/server/index.html>; Katoh & Standley 2013). The concatenated aligned dataset was analyzed separately using maximum likelihood (ML) and Bayesian inference (BI). The best-fit models of evolution for the three loci tested were estimated by MrModeltest V.2.2.

The ML analyses were conducted with RAxML v.7.2.6 (Stamatakis & Alachiotis 2010) using a GTRGAMMA substitution model with 1,000 bootstrap replicates. The robustness of the analyses was evaluated by bootstrap support (MLBS).

Posterior probabilities (PP) (Rannala & Yang 1996; Zhaxybayeva & Gogarten 2002) were determined by Markov chain Monte Carlo sampling (BMCMC) in MrBayes 3.0b4 (Huelsenbeck & Ronquist 2001). Six simultaneous Markov chains were run for 1,000,000 generations and trees were sampled every 100 generations (resulting 10,000 total trees). The first 25% of trees were removed as burn-in phase and the remaining trees were used to calculate posterior probabilities. Posterior probabilities values of the BI analyses (BPP) >0.95 were considered significant. Sequences generated in this study were deposited in GenBank (TABLE 1) and the final matrices and trees in TreeBASE (www.treebase.org; <http://purl.org/phylo/treebase/phylovs/study/TB2:S22878?x-access-code=7f62703b04d4f5d2876667f654bd0398&format=html>).

Phytase activity assessment

Mycelia obtained from fungal strains were inoculated to selective media (formula: calcium phytate 0.1%, dextrose 3.0%, NH_4NO_3 0.5%, KCl 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.003%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.003%, agar 2%, pH 5.5) in a petri dish, and incubated in dark at 25 °C. Strains with transparent circles, indicating phytase activity, were selected for further phytase activity evaluation.

The selected fungi were inoculated in 50 mL volume of liquid medium (formula: dextrose 1.5%, peptone 0.3%, soluble starch 2%, $(\text{NH}_4)_2\text{SO}_4$ 0.2%, KCl 0.05%, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ 0.05%, $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ 0.003%, $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ 0.003%, pH 5.5) in a 250 mL flask with two repeats. The fungi were fermented in a shaker at 28 °C, 160 rpm for 120 hours. The fermentation broth was sampled three times, each sample was centrifuged, and 10 mL of the supernatant were used to evaluate the phytase activity.

Phytase activity was evaluated by spectrophotometry according to the protocol of the Determination of Feed Phytase Activity (GB/T 18634-2009) provided by China National Standardization Administration (CNSA). One unit of phytase activity (U) was expressed as the amount of enzyme that liberates 1 μmol of inorganic phosphorus from a 5.0 mM sodium phytate solution per minute at 37 °C and pH 5.5, while enzyme production was expressed as phytase activity U/mL.

Results

Fungal strain isolation

Two fungal strains representing what proved to be a new species were obtained in this study. One strain JAUCC 1788 was isolated from rice field mud as a saprophyte, and another strain JAUCC 0138 was isolated from the stems of *Acorus calamus* as an endophyte.

Phylogenetic analyses

A multigene phylogeny, based on three loci, was used to infer the relationships between the new taxon and its allied species (FIG. 1). The resulting concatenated aligned dataset comprised 31 isolates representing six genera of

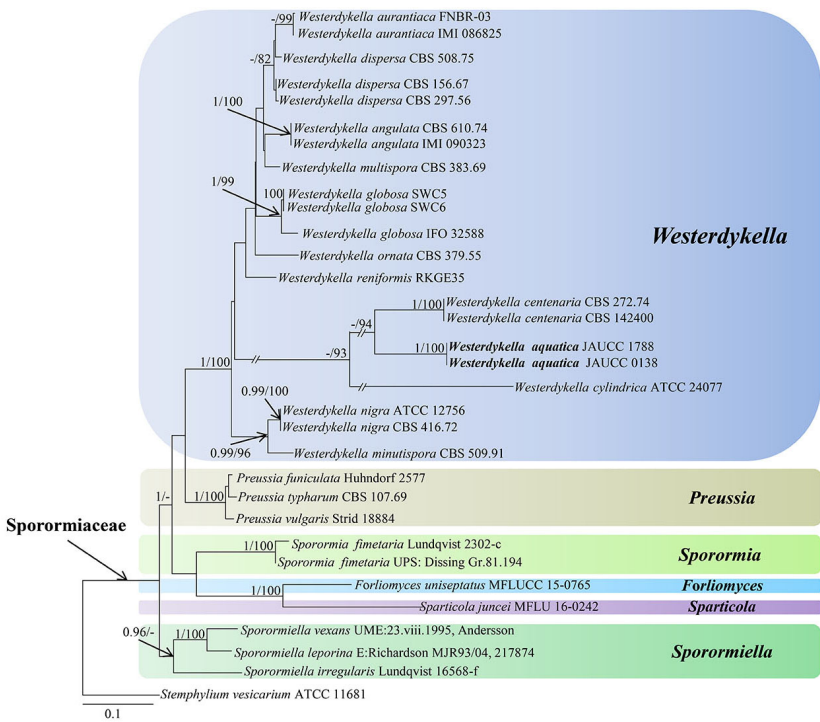


FIG. 1 Consensus tree inferred from a maximum likelihood analysis of ITS, LSU, and β -tubulin gene sequences. The RAXML bootstrap support values (MLBS) and Bayesian posterior probabilities (BPP) are given at the nodes (MLBS/BPP). The tree is rooted with *Stemphylium vesicarium* (ATCC 11681).

Sporormiaceae: *Forliomyces*, *Preussia*, *Sparticola*, *Sporormia*, *Sporormiella*, and *Westerdykella*; and an isolate of *Stemphylium vesicarium* (Wallr.) E.G. Simmons (*Pleosporaceae*) as outgroup. The dataset consisted of 1952 characters (448 for ITS, 815 for LSU, and 689 for β -tubulin, including alignment gaps). The trees generated from ML and Bayesian analyses of the individual loci (data not shown) and the combined dataset showed essentially congruent topologies. The ML tree based on the combined dataset is presented, with bootstrap support values (MLBS) and Bayesian posterior probabilities (BPP) indicated for well supported clades in FIG. 1. Two isolates of the new taxon (*Westerdykella aquatica*) together with other *Westerdykella* species formed a well-supported clade (BPP = 1, MLBS = 100%).

Phytase activity

The phytase activities of the two *Westerdykella aquatica* isolates are presented in TABLE 2.

TABLE 2. Phytase activity of *Westerdykella aquatica* strains.

STRAIN NO.	PHYTASE ACTIVITIES (U/mL)					
		Test 1	Test 2	Test 3	Average	Combined average
JAUCC 0138	Treat 1	0.079	0.114	0.096	0.096±0.014	0.085
	Treat 2	0.070	0.079	0.070	0.073±0.004	
JAUCC 1788	Treat 1	0.079	0.071	0.079	0.076±0.005	0.083
	Treat 2	0.079	0.176	0.018	0.091±0.065	

Taxonomy

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FIG. 2

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Differs from *Westerdykella angulata* and *W. purpurea* by its cylindrical and 3-septate ascospores; from *W. cylindrica* by its light brown ascospores; and from other *Westerdykella* species by its 8-spored asci.

TYPE—China, Jiangxi Province, Jingan County, isolated from mud in a rice field, 15 July 2017, Jun-Bo Zhang (Holotype, HFJAU 0676; ex-type living culture, JAUCC 1788; GenBank MH411090, MH411093, MH424907).

ETYMOLOGY—*aquatica*, referring to the aquatic habitat of the fungus.

COLONIES on PDA medium reaching 90 mm diam after 3 wk at 25 °C, spreading, flat, velvety, with sparse aerial mycelium, yellow, reverse dark yellow, slightly zonate, with several white rings, surface and margins smooth. Hyphae 1.4–4 µm diam, septate, branched, hyaline to light brown. SEXUAL STATE: Cleistothecia scattered, superficial, glabrous, globose, black, c. 100–260 µm diam. Peridium thin, membranous, composed of a single layer of globose to ellipsoidal, angular cells. Asci initials somewhat ovoid, later becoming globose to subglobose, 14–17 × 12–16 (mean = 15.3 × 14.1 µm, n = 30), persistent, 8-spored. Ascospores 17–38 × 2.5–4 µm (mean = 27 × 3 µm, n = 30), cylindrical, with rounded ends, 3-septate, slightly constricted at the septa, mostly separated into four segments at the very early stage of spore-formation in asci. Ascospore segments 4.5–11 × 2.5–3.5 µm (mean = 6.5 × 2.9 µm, n = 50), ellipsoidal, subhyaline, smooth-walled, light brown, mostly with a big guttule at each end, no germ-slits observed. ASEXUAL STATE: not observed.

ADDITIONAL SPECIMEN EXAMINED—CHINA, JIANGXI PROVINCE, Jiangxi Agricultural University, endophytic in *Acorus calamus* L. (*Acoraceae*) in a pond, 21 March 2014, Guan-Xiu Guan (HFJAU 0677; living culture, JAUCC 0138; GenBank MH411091, MH411092, MH424906).

Discussion

Westerdykella aquatica is characterized by its globose cleistothecia, globose to subglobose, persistent and 8-spored asci, and cylindrical, 3-septate, light brown ascospores. These characters fit well within the generic concept of *Westerdykella*. Furthermore, in the phylogenetic tree inferred from ITS, LSU, and β -tubulin genes, *W. aquatica* and other 19 reference strains of 11 species of *Westerdykella* form a well-supported clade (BPP = 1, MLBS = 100%).

Westerdykella aquatica resembles *W. angulata*, *W. cylindrica* and *W. purpurea* in having 8-spored asci. However, *W. aquatica* differs from *W. angulata* and *W. purpurea* in the shape of its ascospores. The ascospores of *W. aquatica* are cylindrical and 3-septate, while those of *W. angulata* and *W. purpurea* are angular (Das 1962; von Arx 1975; Kruys & Wedin 2009). *W. cylindrica* differs from *W. aquatica* in its reddish brown ascospores (Malloch & Cain 1972). Furthermore, the strains of *W. cylindrica* and *W. aquatica* are well separated in the phylogenetic tree (Fig. 1).

Although various chemical compounds have been reported from species of *Westerdykella* (Lee & al. 1997, 1999; Ebead & al. 2012; Xu & al. 2017a,b), our study is the first report of phytase production by the genus. Richardson (1994) reported that 20–80% of P in soils is found in the organic form, of which phytate (inositol hexaphosphate) is usually a major component. The benefits of phytase are its double effects on reducing the use of expensive inorganic phosphorus as fertilizer and its reduction of environmental pollution from excessive manure phosphorus runoff (Wang & Yang 2007). *Westerdykella aquatica* JAUCC 1788 was isolated from the mud of rice field, and its presence may help liberate P from the soil and reduce the use of P fertilizer.

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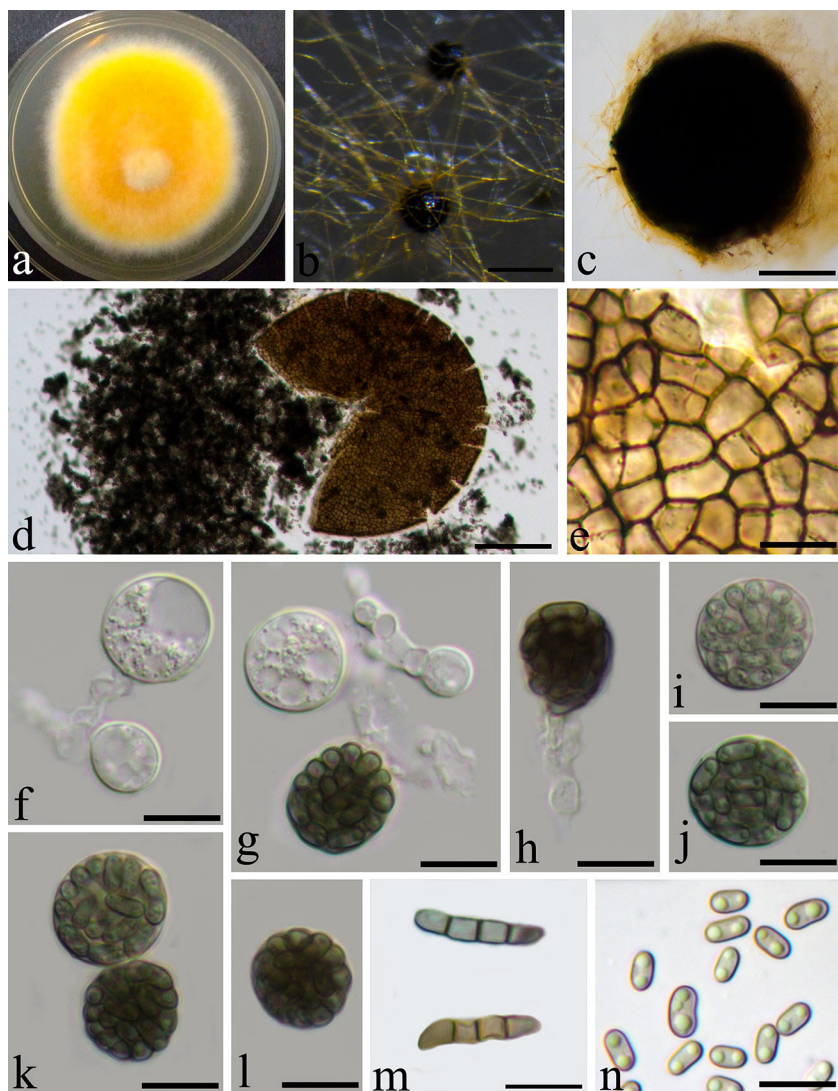


FIG. 2. *Westerdykella aquatica* (holotype, HFJAU 0676). a. Colony on PDA; b. Ascoma on PDA; c. Ascoma; d. Squashed ascoma; e. Peridium; f. Immature ascus; g–l. Asci; m. Ascospores; n. Ascospore segments. Scale bars: b = 1 mm; c, d = 100 µm; e–n = 10 µm.

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